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Smooth muscle myosin: regulation and properties

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The relationship of the biochemical states to the mechanical events in contraction of smooth muscle cross-bridges is reviewed. These studies use direct measurements of the kinetics of P_i and ADP release. The rate of release of P_i from thiophosphorylated cycling cross-bridges held isometric was biphasic with turnovers of 1.8 s^{-1} and 0.3 s^{-1}, reflecting properties and forces directly acting on cross-bridges through mechanisms such as positive strain and inhibition by high-affinity MgADP binding. Fluorescent transients reporting release of an ADP analogue 3′-deac-edaADP were significantly faster in phasic than in tonic smooth muscles. Thiophosphorylation of myosin regulatory light chains (RLCs) increased and positive strain decreased the release rate around twofold. The rates of ADP release from rigor cross-bridges and the steady-state P_i release from cycling isometric cross-bridges are similar, indicating that the ADP-release step or an isomerization preceding it may limit the ATPase rate. Thus ADP release in phasic and tonic smooth muscles is a regulated step with strain- and dephosphorylation-dependence. High affinity of cross-bridges for ADP and slow ADP release prolong the fraction of the duty cycle occupied by strongly bound AM·ADP state(s) and contribute to the high economy of force that is characteristic of smooth muscle. RLC thiophosphorylation led to structural changes in smooth muscle cross-bridges consistent with our findings that thiophosphorylation and strain modulate product release.

Keywords: smooth muscle; cross-bridge cycle; adenosine diphosphate; actomyosin; myosin phosphorylation

1. INTRODUCTION

The ability to maintain force at low levels of ATPase activity and shortening velocity is a major characteristic of smooth muscle extending from mammalian to invertebrate systems. Vertebrate smooth muscle is normally activated by RLC phosphorylation and the force generated can be maintained at low phosphorylation levels. However, force is not redeveloped upon quick release after phosphorylation has declined to low levels (Somlyo *et al.* 1988). This high force–low phosphorylation state (catch-like state, also known as latch) occurs in smooth muscle after a decline in Ca^{2+} concentration that initiates dephosphorylation of RLC (Dillion *et al.* 1981). The high affinity of smooth muscle myosin for ADP (Vyas *et al.* 1992; Fuglsang *et al.* 1993; Nishiye *et al.* 1993; Butler & Siegman 1998; Cremo & Geeves 1998; Gollub *et al.* 1999), cycling of dephosphorylated cross-bridges (Vyas *et al.* 1992; Butler & Siegman 1998; Somlyo *et al.* 1988) and the effects of strain and RLC thiophosphorylation on product release (Khromov *et al.* 2004) contribute to this catch-like state and will be one focus of this review along with a survey of some of the structure-linked changes evident in smooth muscle myosin.

2. PHOSPHATE RELEASE KINETICS AND FORCE DEVELOPMENT

A time-dependent decline in cross-bridge actomyosin ATPase activity occurs during maintained contraction in smooth muscles whose RLCs are thiophosphorylated (He *et al.* 1998). Thiophosphorylated RLCs are functionally equivalent to phosphorylated RLCs but are resistant to protein phosphatase activity. It follows that under appropriate conditions change in ATPase activity is independent of RLC phosphorylation. This may be demonstrated in time-resolved measurements of P_i release by cycling cross-bridges (He *et al.* 1998) using the P_i-reporter, MDCC-PBP (Brune *et al.* 1998). The fluorescence of MDCC-PBP increases upon binding P_i and this enables

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‡Deceased 14 January 2004. The UK authors dedicate this paper to our co-author Professor Andrew Somlyo who died in early 2004 and extend great sympathy to our colleagues at the University of Virginia. Andrew was a pre-eminent scholar in smooth muscle and a dedicated colleague and friend whose many visits to our laboratories were always a time of great scientific stimulation. He was founding Chairman of The Pennsylvania Muscle Institute and of The Department of Molecular Physiology and Biological Physics, University of Virginia, a post held at the time of his death.

One contribution of 14 to a Discussion Meeting Issue 'Myosin, muscle and motility'.

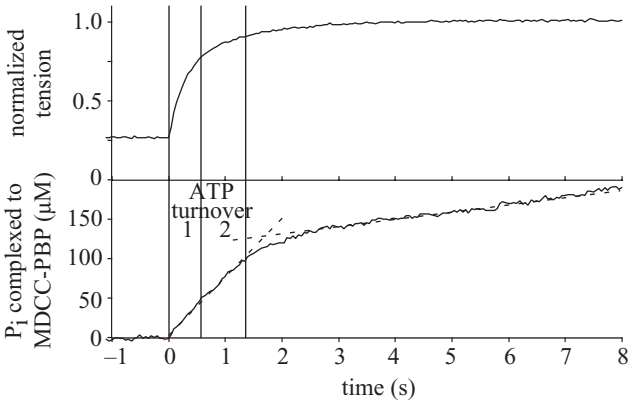

Figure 1. Average time course of P_i release and tension development after photolysis of caged ATP in triton-permeabilized portal vein muscles previously thiophosphorylated (RLC thiophosphorylation greater than 80%; $n = 7$). Fluorescence increased with an initial fast phase of 80 μM s^{-1}, followed by a slow phase of 13.7 μM s^{-1}. Based on 52 μM myosin heads measured in this smooth muscle, tension had reached 72% of maximal tension by the end of the first cross-bridge turnover. The fast phase of P_i release encompassed about two turnovers of the myosin heads. No phosphate burst was detectable before force development. The contribution of the calcium-independent ATP breakdown has been subtracted from the traces. (From He *et al.* (1998) with permission.)

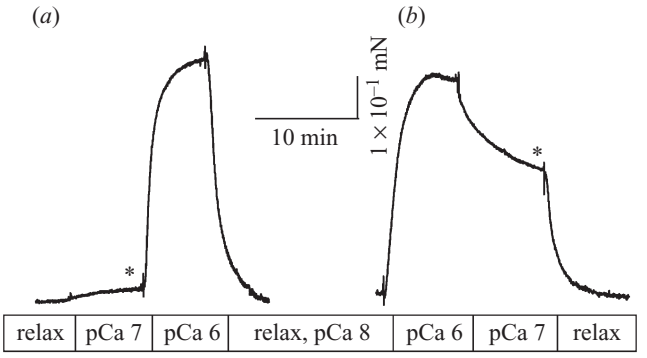

Figure 2. Force hysteresis. (*a*) Ca-ascending protocol; the permeabilized femoral artery strip was activated by transfer from relaxing to a pCa 7.0 solution containing 2.0 mM MgATP, zero CP followed by activation in a pCa 6.0 solution. At the plateau of force, the strip was relaxed by transfer to a pCa 8.0 solution. (*b*) Ca-descending protocol; the strip was first activated in a pCa 6.0 solution followed by transfer into a pCa 7.0 solution. Note the different level of force at pCa 7.0 in the two traces (denoted by an asterisk). (From Khromov *et al.* (1998*a*) with permission.)

the kinetics of P_i release to be monitored with micromolar sensitivity and millisecond time resolution. Synchronous cross-bridge cycling may be initiated by releasing ATP rapidly on photolysis of caged ATP into triton-permeabilized smooth muscle.

Figure 1 shows that, upon photolytic release of ATP into rigor muscle in which the RLCs had been thiophosphorylated, MDCC-PCP fluorescence increases. P_i release exhibits an initial fast phase with a rate of 80 (±7.6 s.e.m.) μM^{-1} s^{-1} followed by a 5.8-fold slower steady-state phase of 13.7 (±1.1) μM^{-1} s^{-1} (He *et al.* 1998). Based on the measured myosin head concentration of 52 μM and assuming 84% thiophosphorylation, these rates correspond to 1.8 s^{-1} during the first and second turnovers and 0.3 s^{-1} during the third turnover, which we treat as being the steady-state rate. The initial rate of cross-bridge cycling in this portal vein smooth muscle is comparable to the actin-activated ATPase activity of gizzard HMM in solution (1.9 s^{-1}; Sellers 1985). The force increment was essentially complete by the end of the fast phase (figure 1).

In smooth muscle with non-thiophosphorylated RLC, the rate of force development (Horiuti *et al.* 1989) and actomyosin ATPase activity are limited by the extent of RLC phosphorylation. The initial rate of P_i release (1.0 (±0.1) μM s^{-1}) and force development were significantly slower under these conditions, but overall P_i was still biphasic also falling to 0.3 μM s^{-1}. Activities of myosin light chain kinase and phosphatase ('pseudo-ATPase') contribute an upper limit of 20% to the ATP usage during cross-bridge cycling (He *et al.* 1998). Another upper limit of pseudo-ATPase activity can be based on a myosin light chain phosphatase activity of *ca.* 0.07 s^{-1}, because the former cannot exceed the activity of the latter (Khromov *et al.* 1995). When 57% of the 52 μM heads are phosphorylated during a peak contraction, the pseudo-ATPase activity would account for ATP breakdown of 0.07 s^{-1} × 30 μM phosphorylated heads, 2 μM s^{-1}, or 4% of the total P_i release rate (51 μM s^{-1}). Estimates of less than 20% have also been reached by others (Butler *et al.* 1986; Driska *et al.* 1989; Paul 1989).

Thus the transition from a fast to slow P_i release is not a result of a decline in RLC phosphorylation. This raises questions as to which force-generating states of the cross-bridge cycle are involved in the transition, what is their biochemical nature and what factors contribute to slowing the ATPase rate? We have little to contribute to the first two questions at this stage other than to suggest that the states involved are force-generating actomyosin–ADP states with or without bound P_i. The factors may include inhibition by MgADP as *ca.* 50 μM is released on the first turnover. Positive strain on the cross-bridges may also contribute to the slowing of the cycle. We have suggested that, given the high ratio of actin to myosin filaments in smooth muscle (Ashton *et al.* 1975), it is possible that following the initial attachment of cross-bridges, at least some of the ATPase breakdown occurs through the slower associated state hydrolysis pathway (White *et al.* 1997) and/or cooperative cycling of non-phosphorylated myosin (Himpens *et al.* 1988; Somlyo *et al.* 1988; Vyas *et al.* 1992; Butler & Siegman 1998).

The approximately three- to fivefold difference between fast and slow rates of P_i release in permeabilized smooth muscles with either Ca^{2+}–calmodulin-dependent RLC phosphorylation (He *et al.* 1998) or RLC thiophosphorylation is similar to time-dependent transitions in energy consumption correlated with force development to lower consumption during force maintenance (Siegman *et al.* 1980).

Similar nonlinear time courses of P_i release occur in skeletal muscle where the issue of comparable RLC phosphorylation does not arise. A recent study (West *et al.* 2004) investigated the fivefold decrease in the P_i release rate during isometric contraction and compared it with the similar decrease in the rate of energy release at the beginning of isometric tetani in isolated muscle or fibres. In both situations

Table 1. Kinetic parameters of the fluorescence change following displacement of 3′-deac-edaADP in skeletal and smooth muscles.

	fluorescence			
specimen	direction (±)	amplitude[a] (% ± s.e.m.)	rate constant (s^{-1})(s.e.m.)	*n*
skeletal fibre	+	25±10	40±5	3
unstrained smooth muscle Rfa				
(unphosphorylated)	−	2±1	0.29±0.03	16
(thiophosphorylated)	−	2±1	0.63±0.08	12
strained smooth muscle Rfa				
(unphosphorylated)	−	2±1	0.14±0.09	10
(thiophosphorylated)	−	2±1	0.43±0.04	10
unstrained smooth muscle Rbl				
(unphosphorylated)	−	4±1	0.57±0.06	18
(thiophosphorylated)	−	4±1	1.4±0.2	16
strained smooth muscle Rbl				
(unphosphorylated)	−	4±1	0.3±0.2	15
(thiophosphorylated)	−	3±1	0.5±0.1	9

[a] Change in fluorescence relative to fluorescence before photolysis of caged ADP (Khromov *et al.* 2004).

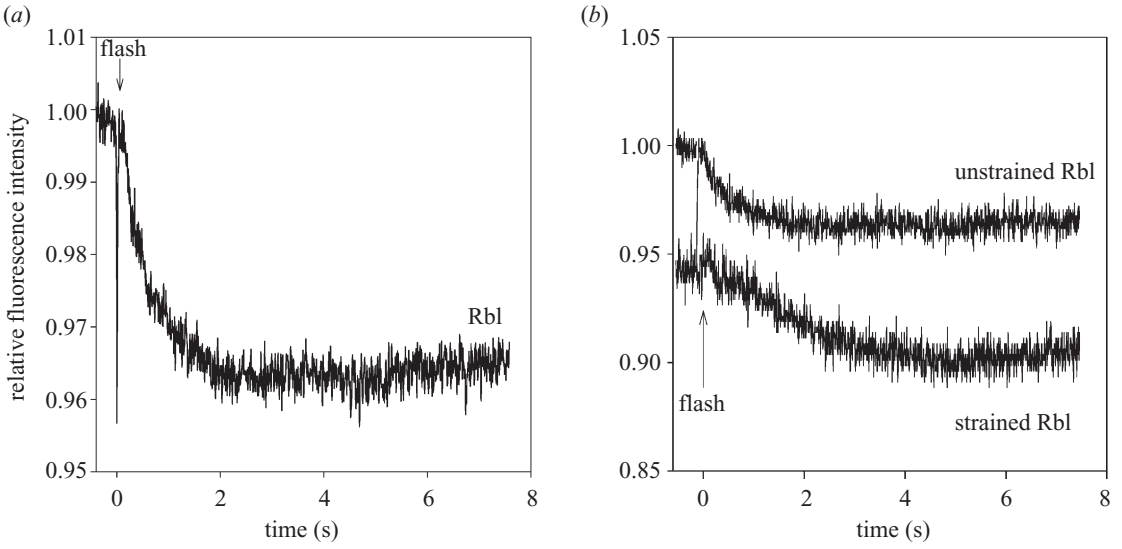

Figure 3. (*a*) Typical record of relative decrease in fluorescence (relative to fluorescence before photolysis) of smooth muscle Rbl loaded with 3′-deac-edaADP (4 μM) initiated by photolysis of 2 mM caged ADP. (*b*) The time course of relative fluorescence decrease after photolysis of caged ADP in the unstrained or strained (up to *ca*. 30% of P_{max}) Rbl smooth muscle. For clarity the strained trace was shifted down by 0.05 units. (From Khromov *et al.* (2004) with permission.)

the decline in the rate of ATP hydrolysis could be attributed to several factors: high ATPase activity at the beginning of the contraction associated with stretching of the series compliance resulting in sarcomere shortening, changes in relative ATP, ADP and P_i concentrations, and in the distribution of cross-bridge states towards AM·ADP becoming more dominant by virtue of the slow rate of ADP release in isometric muscle (He *et al.* 1998; West *et al.* 2004).

3. MAGNESIUM ADENOSINE DIPHOSPHATE IN FORCE MAINTENANCE OF NON-PHOSPHORYLATED CROSS-BRIDGES IN PHASIC AND TONIC SMOOTH MUSCLE

The rate of unloaded shortening velocity of smooth and striated muscles is limited by ADP release (Siemankowski *et al.* 1985). Demonstration of the high affinity of MgADP for smooth muscle actomyosin pointed to a potentially important role of a strongly bound AM·ADP state during isometric force maintenance of smooth muscle. The high affinity of MgADP for smooth muscle myosin is detectable *in situ* (Fuglsang *et al.* 1993; Nishiye *et al.* 1993; Gollub *et al.* 1999; Khromov *et al.* 2004) and in solution (Cremo & Geeves 1998). Our early studies estimated the rate constant of MgADP release indirectly, based on the concentration-dependent effect of ADP on the kinetics of relaxation upon photolysis of caged ATP in smooth muscles in the rigor state (Fuglsang *et al.* 1993; Nishiye *et al.* 1993).

We have also used other experimental paradigms to show that MgADP promotes a catch-like state developed

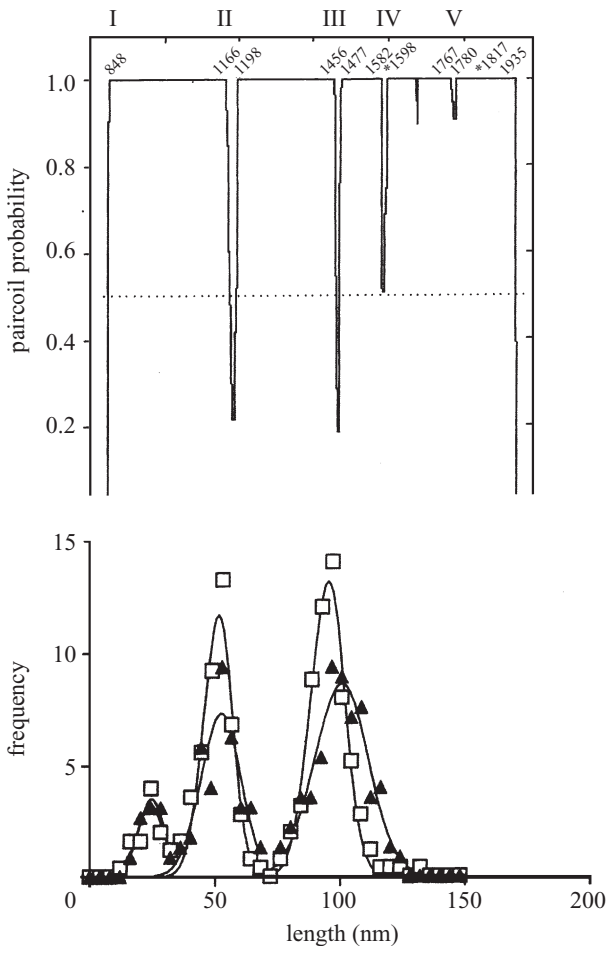

Figure 4. (*a*) Probability of coiled-coil structure in the tail region of myosin using a paircoil probability program (Berger *et al.* 1995). The residue numbers are indicated along the top of the graph, as well as the skip residues at 1199, 1592 and 1817 indicated by an asterisk. Regions of low probability for coiled-coil structure are indicated by Roman numerals. Based on a helical rise of 0.148 nm per residue for an α-helical coiled-coil, the bend at position 2 would occur at 48–51 nm from proline 848, and this has been aligned with the data in (*b*). Myosin sequence from Yanagisawa *et al.* (1987). (*b*) Frequency distribution of the position of the bends in the tail of non-phosphorylated (triangles) and thiophosphorylated (squares) myosin. The zero position on the abscissa represents the head–neck junction. (From Zhang *et al.* (1997) with permission.)

through force-calcium hysteresis in tonic smooth muscle (Khromov *et al.* 1998*a*). In this hysteresis protocol (Moreland & Murphy 1986), the force development by a small increment in Ca^{2+} concentration (pCa 7.0) from a nominally Ca^{2+}-free condition is significantly lower than the force reached by descending from relatively high Ca^{2+} (pCa 6.0) to the identical low level of Ca^{2+} (pCa 7.0; figure 2). This force-calcium hysteresis was observed at a low MgATP to MgADP concentration ratio (2 mM MgATP, zero CP and CK), but not at higher relative ATP concentration (10 mM MgATP, low free $[Mg^{2+}]$ plus exogenous CP and CK). The velocity of unloaded shortening, V_0, at pCa 7.0 in this Rfa-permeabilized preparation was not significantly different whether the ascending or the descending protocol was used; $V_0 = 0.020 \pm 0.004$ and 0.020 ± 0.006 lengths s^{-1}, respectively. At pCa 6.0, V_0 was significantly higher, 0.07 ± 0.01 lengths s^{-1}. The level of RLC phosphorylation was $10 \pm 2\%$ at pCa 7.2, reached $70 \pm 15\%$ with increasing calcium to pCa 6.0 and returned to its low level ($9 \pm 2\%$) when Ca^{2+} was decreased at a time when force and the ratio of stiffness:force were significantly higher (54% versus 3%) under this calcium-descending than the calcium-ascending protocol. Unlike the tonic femoral artery, phasic smooth muscle does not exhibit significant hysteresis under the same experimental conditions. Exogenous CP (10 mM) applied during the descending protocol in Rfa reduced endogenous [MgADP] to 46 ± 10 μM and abolished force hysteresis. Thus a force hysteresis is observed in smooth muscle when $[Ca^{2+}]$ is reduced from a high to an intermediate concentration at relatively low, but physiological [MgATP]/[MgADP] ratios. We conclude that this hysteresis is primarily caused by the development of a population of strained, non-phosphorylated cross-bridges having high affinity for MgADP.

Similar conclusions were reached when relaxation from isometric tension was initiated by reducing free $[Ca^{2+}]$ through photolysis of the caged photolabile Ca^{2+} chelator diazo-2 in the presence and absence of CP and variable MgADP concentrations (Khromov *et al.* 1995). Upon photolysis of diazo-2, RLC phosphorylation decayed with a rate constant of 0.07 ± 0.02 s^{-1}, reaching basal levels at a time when force had fallen only 40%. The slowing effect owing to the presence of MgADP on the post-dephos phorylation phase of relaxation was much more prominent in tonic than in phasic smooth muscle. The effect of MgADP is a result of its competition with MgATP in binding to cross-bridges, so increasing the population of force-producing non-phosphorylated AM·ADP states.

4. ADENOSINE DIPHOSPHATE RELEASE AND ITS MODULATION BY STRAIN AND REGULATORY LIGHT CHAIN PHOSPHORYLATION USING A FLUORESCENT NUCLEOTIDE

Direct measurements of ADP release with radioisotope-labelled nucleotides have been made in permeabilized smooth muscle (reviewed in Butler & Siegman 1998). Effects of MgADP on cross-bridge orientation (e.g. Gollub *et al.* 1996, 1999) and on rigor force (e.g. Nishiye *et al.* 1993; Dantzig *et al.* 1999; Khromov *et al.* 2001) have been used to estimate ADP binding. Here, we describe studies with fluorescent nucleotide probes.

Fluorescent nucleotide analogues such as mant-ATP (Hiratsuka 1983) have been used to probe nucleotide binding to skeletal muscle myosin and actomyosin (Bauer *et al.* 1997; Chaen *et al.* 1997). The fluorescent ATP analogue 3′-deac-edaATP is labelled with a coumarin and is a substrate for myosin and actomyosin (Webb & Corrie 2001). 3′-deac-edaATP(ADP) has excitation and emission maxima at 430 nm and 477 nm, respectively, and so its fluorescence in permeabilized muscle may be observed without interference from endogenous fluorescence.

Initial experiments establishing the validity of using the analogue were done with skeletal muscle proteins. 3′-deac-edaADP has fluorescence quantum yields in free solution and bound to skeletal S1 of 0.038 and 0.012, respectively. S1 triphosphatase activity and the diphosphate dissociation rate were very similar to those of the natural nucleotide

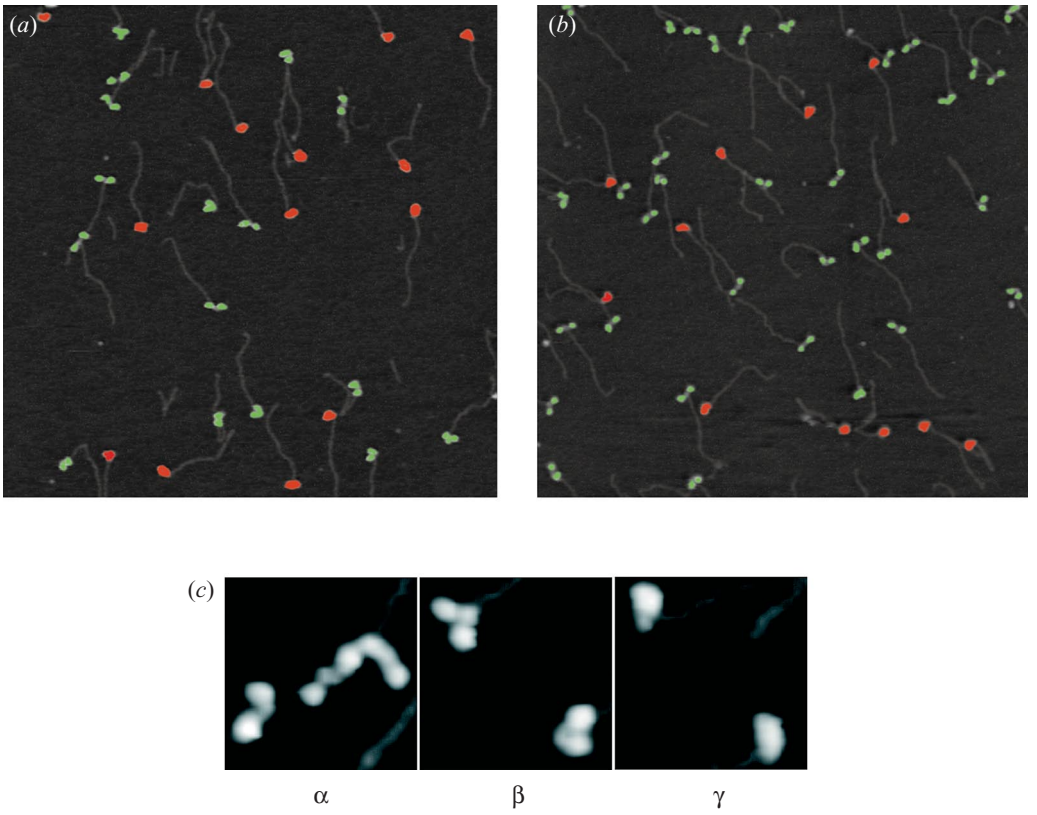

Figure 5. (*a*,*b*) Cryo-atomic force microscopy images of non-phosphorylated and thiophosphorylated myosin on a positively charged mica surface illustrating that phosphorylation of the RLCs increases the open conformation of the two heads of smooth muscle myosin. Both samples were prepared in an aqueous solution containing 0.3 M KCl, 10 mM KH_2PO_4, 1 mM EGTA 1 mM EDTA and 1 mM dithiothreitol at pH 6.8. In the case of non-phosphorylated myosin (*a*), the relative partitioning of the myosin heads into the split and closed conformation remains unchanged compared with a negatively charged mica surface, indicating that the charge on the substrate does not have a major influence on the head arrangement. Thiophosphorylated myosin (*b*) shifted the distribution into α and β configurations with a 50% increase in the open confirmation. To show clearly the shift in distribution, the α and β forms are green and the γ is red. Image size *ca*. 1 μm. (*c*) Examples of three different categories of myosin molecule conformations. α and β are essentially open conformations in which the two heads are separated. In the γ conformation the two heads could not be distinguished in the image, suggesting that the two heads of these molecules could be in close apposition. (From Sheng *et al.* (2003) with permission.)

(Webb & Corrie 2001). Further evidence supporting the interpretation that the 3′-deac-edaADP fluorescence signal is reporting its binding and unbinding from myosin is based on imaging studies (Khromov *et al.* 2004). Single rabbit psoas fibres loaded with 3′-deac-edaADP were imaged before and after photolysis of caged ADP using 2-photon excitation of 3′-deac-edaADP fluorescence at 760 nm and imaging at 470–490 nm. The fluorescent striation pattern disappeared after photolytic release of ADP, indicating displacement of the 3′-deac-edaADP from the A-bands.

With the background work done on skeletal muscle proteins, we are now in a position to probe directly the effects of physiologically relevant parameters such as strain and RLC phosphorylation on ADP release from myosin in solution and in permeabilized smooth muscle bundles (Khromov *et al.* 2004).

Thiophosphorylation of gizzard smooth muscle myosin RLC increased the myosin ATPase rate to 0.04 s^{-1} versus 0.007 s^{-1} for non-phosphorylated, but did not significantly change either the amplitudes or the rate constants of the fluorescence decrease after displacement of 3′-deac-edaADP, which were about two orders of magnitude faster (see below). This also confirms ADP release is not the rate-limiting step of the ATPase in solution (Marston & Taylor 1980; Rosenfeld & Taylor 1984; Siemankowski *et al.* 1985; Rosenfeld *et al.* 1998).

Fluorescent transients of isolated non-phosphorylated and thiophosphorylated smooth muscle myosin and skeletal HMM with bound 3′-deac-edaADP were monitored after displacement by ADP upon photolysis of caged ADP. The relative amplitudes and rates of these changes were different for the two myosins: positive *ca*. 15% at *ca*. 3 s^{-1} for skeletal HMM and negative *ca*. 6% at *ca*. 1–2 s^{-1} for smooth muscle myosin, respectively. The difference in polarity of the fluorescence signal upon displacement of 3′-deac-edaADP may reflect the different atomic structures of the nucleotide binding pockets and/or the associated loop 1 (Sweeney 1998) of skeletal and smooth muscle.

We now turn to permeabilized muscle bundles. The velocity of unloaded shortening, an index of smooth muscle actomyosin ATPase activity, indicated that 3′-deac-edaADP, when compared with the natural nucleotide, was a satisfactory substrate for smooth muscle contraction. 3′-deac-edaADP is, at best, a very poor substrate for myosin light chain kinase and CK. The experimental apparatus and optimal conditions for 3′-deac-edaADP loading of smooth muscle bundles have been described in detail (Khromov *et al.* 2004).

Different properties of phasic (fast) and tonic (slow) smooth muscles are inherent to their myosin isoforms (Horiuti *et al.* 1989) and are apparent in the faster rate of force development in phasic than tonic muscles with thiophosphorylated RLCs. It was therefore of interest to compare ADP release rates of the two classes of smooth muscle. The effects of strain and myosin RLC phosphorylation on the kinetics of 3′-deac-edaADP, and hence ADP release from rigor cross-bridges in phasic (Rbl) and tonic (Rfa) smooth muscle, were determined by monitoring fluorescence transients. At zero imposed strain in both tonic and phasic smooth muscles, fluorescence decreased monotonically on displacement of 3′-deac-edaADP by ADP generated by caged ADP photolysis (figure 3*a*) similar to that observed for smooth muscle myosin in solution. The rate of fluorescence decrease was significantly faster in the phasic (Rbl) compared with the tonic (Rfa) smooth muscle: 0.57 ± 0.06 s^{-1} for Rbl and 0.29 ± 0.03 s^{-1} for Rfa (table 1). Rates were reduced twofold by positive strain (*ca.* 25–30% of P_{max}) imposed on the muscles to 0.3 ± 0.2 s^{-1} for Rbl and 0.14 ± 0.09 s^{-1} for Rfa (figure 3*b*; table 1).

Thiophosphorylation of RLCs increased the release rate of ADP (two- to threefold) in both phasic and tonic smooth muscles compared with muscles with non-phosphorylated RLCs (table 1), indicating that ADP release is a regulated step. This contrasts with the absence of an effect of thiophosphorylation on 3′-deac-edaADP release from smooth muscle myosin in solution. This probably reflects the greater steric constraints and protein–protein interactions imposed on the ADP binding site in the intact muscle. We also note that these effects may be in part responsible for the much slower ADP release (table 1) than from actomyosin in solution (Marston & Taylor 1980; Rosenfeld & Taylor 1984; Siemankowski *et al.* 1985; Rosenfeld *et al.* 1998) and laser traps (Baker *et al.* 2003), although slower ADP release also occurs in isometric (as here) compared with shortening muscle for which solution studies are a better model.

Studies of the ADP-induced rotation of the light chain domain of smooth muscle upon RLC thiophosphorylation indicated an apparent K_d about seven times greater than the K_d measured in non-phosphorylated preparations, while thiophosphorylation of smooth muscle S1 did not alter the MgADP affinity (Gollub *et al.* 1999). This is interpreted by the authors as a strain-dependent stabilization delaying ADP release in smooth muscle.

It is interesting to relate the two phases of P_i release from rabbit portal vein (figure 1) with 3′-deac-edaADP release rates (table 1) also from thiophosphorylated muscles. The second phase of P_i release of 0.3 s^{-1} probably reflects the steady-state ATPase rate in a strained state and is one- to twofold less than the ADP analogue-release rate for the strained Rbl and Rfa at 0.5 s^{-1} and 0.43 s^{-1}, respectively. The initial phase of P_i release at 1.8 (± 0.1) s^{-1} was only slightly faster than the ADP analogue-release rate of 1.4 (± 0.2) s^{-1} in unstrained Rbl. In analysing these data we first need to consider whether the transient phase of P_i release occurs because a later step in the ATPase pathway such as ADP release is rate limiting. However, ADP release being rate limiting cannot be the whole story, as otherwise there would be an additional fast phase significantly greater than 1.4 s^{-1} representing P_i release during the first turnover of the ATPase. The concentration of this fast phase would be less than that of the ATPase active site concentration of *ca.* 50 μM.

As referred to above, the origin of the transient phase in skeletal muscle has been discussed in detail (West *et al.* 2004 and references therein). We consider that it is at least plausible from our data that ADP release is partly rate limiting in both phases of the smooth-muscle P_i release experiment (figure 1). It is evident that strain is an important factor; in our ADP experiments the muscles were less strained (only 30% P_{max}) than during the steady-state contraction. Even so 1.4- to 2.8-fold reductions in rate were recorded as strain was applied (table 1). Retardation of the steady-state ATPase with strain as inferred by our 3′-deac-edaADP release kinetics may contribute to a possible Fenn effect (Fenn 1923) in smooth muscle. A parallel inference was also made from single molecule studies with smooth muscle S1 (Veigel *et al.* 2003).

Thus development of this ADP-analogue labelled with fluorescent coumarin has enabled us to probe directly the effects of physiologically relevant parameters and so investigate further molecular processes of smooth muscle *in situ*.

5. STRUCTURAL AND MECHANICAL CHANGES INDUCED BY REGULATORY LIGHT CHAIN PHOSPHORYLATION AND ADENOSINE DIPHOSPHATE

Thiophosphorylation of the RLCs accelerates ADP release from smooth muscles in the rigor state (table 1), implying that it produces a structural change in these non-cycling cross-bridges attached to actin. Thiophosphorylation of the RLC increases rigor stiffness of Rbl smooth muscle (Khromov *et al.* 1998*b*). We ascribe this to a change in the linear component of rigor stiffness residing within the myosin molecule because of the high specificity of myosin light chain kinase for RLC. The difference in stiffness in the non-phosphorylated and phosphorylated muscles is unlikely to represent a difference in the number of attached cross-bridges. This is thought to be near 100% and is reflected in the equal amplitudes of rigor force in the thiophosphorylated and non-thiophosphorylated muscles (Khromov *et al.* 1998*b*).

The increase in stiffness owing to RLC phosphorylation could also occur in the lever arm where electrical birefringence measurements indicate a contribution of the RLC to the stiffness of the C-terminus of the long α-helix in S1 (Eden & Highsmith 1997). There are interesting structural homologies and comparisons that can be made between calmodulin and myosin light chains (Rayment *et al.* 1993; Houdusse & Cohen 1996). Calmodulin has great propensity to undergo conformational changes such as occur when it interacts with myosin light chain kinase in response to calcium (Krueger *et al.* 1998). We have proposed that a phosphorylation-induced tightening of structure could occur by the N-terminal (phosphorylation site) region of the RLC wrapping around the exposed myosin heavy chain α-helix. This in turn was postulated to increase muscle stiffness (Khromov *et al.* 1998*b*).

Structure changes more distal to the myosin head are also phosphorylation dependent. Thus such phosphorylation-induced changes in myosin were observed in studies using cryo-atomic force microscopy to image thiophosphorylated and non-thiophosphorylated smooth muscle

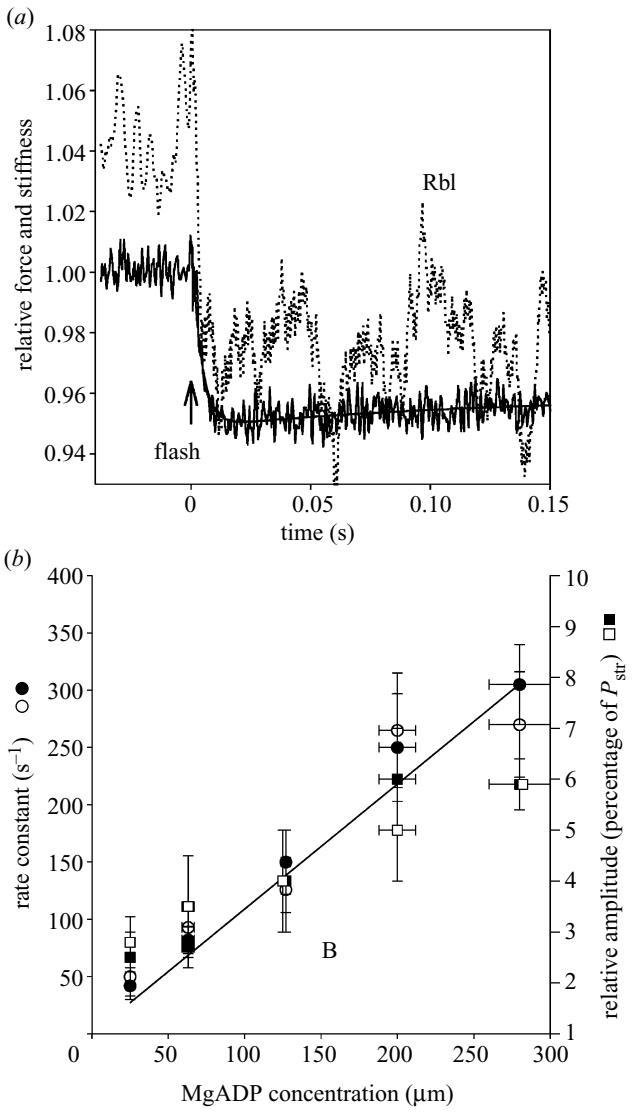

Figure 6. (*a*) Original records showing the decrease in rigor force and stiffness after photolysis of caged ADP in Rbl smooth muscle in the zero Ca high rigor state following the standard protocol. The muscle was stretched up to 0.7 P_{max} before photolysis of 2 mM caged ADP that generated *ca*. 0.2 mM MgADP. The relative decreases in rigor force and stiffness (dotted line) were *ca*. 5% and *ca*. 6%, respectively. The data were normalized to values before photolysis. The trace of the stiffness signal was artificially lifted by *ca*. 0.4 units for reasons of clarity. The result of fitting to two exponentials over 0.2 s is shown as a smooth line through the force record, and gave a rate constant for the faster phase of 200 s^{-1} and 5.2% amplitude. (*b*) The [MgADP] dependence of the rate constants (filled circles) and amplitudes (filled squares, expressed as percentage of post-stretch rigor force P_{str}) of MgADP-induced decrease in rigor force in non-phosphorylated Rbl smooth muscle. The strips were stretched up to 0.6–1.0 of P_{max} before photolysis of caged ADP. The open circles and open squares are rate constants and amplitudes of force responses to photolysis of caged ADP having thiophosphorylated RLC. The number of experiments at each [MgADP] was $n = 4$–7. The line is drawn for the rate constant and is constrained to pass through the origin. (From Khromov *et al.* (2001) with permission.)

myosin molecules. A significant shortening was observed in the hinge region at the juncture of subfragment 2 and light meromyosin (figure 4), thought to reflect a helix-coil transition (Zhang *et al.* 1997), as has been proposed for skeletal muscle myosin under different conditions (Harrington 1971). The tail length of skeletal myosin has also been shown to decrease by 22 nm over a 17 °C increase in temperature (Walker *et al.* 1991). Interestingly, phosphorylation of the RLCs of invertebrate and vertebrate myosin filaments leads to marked disordering of the filament backbone and increased mobility of the myosin heads which move away from the backbone (Levine *et al.* 1995). Thus, phosphorylation of the RLCs of smooth muscle may, in addition to switching on the motor, also induced a α-helix to random coil transition in the tail and movement of the heads away from the myosin filament backbone. However, we have no direct evidence that either of these processes affect myosin compliance.

These findings that thiophosphorylation of RLC induces increased stiffness of smooth muscle in rigor (Khromov *et al.* 1998*b*) as well as the increase in the separation of the two heads of the single myosin molecule imaged by cryo-atomic force microscopy (figure 5; Zhang *et al.* 1997; Sheng *et al.* 2003) are consistent with our findings of an effect of RLC thiophosphorylation on ADP release from non-cycling (rigor) cross-bridges. This implies that this modification of the RLC has a structural effect on the myosin head, independent of dissociation of myosin from actin and catalytic activity.

ADP also causes structure changes in smooth muscle myosin. Addition of MgADP to smooth muscle in a high-tension rigor state induces functional and structural changes independent of the state of RLC phosphorylation or strain (Gollub *et al.* 1999; Khromov *et al.* 2001). This is consistent with the axial rotation observed in cryo-electron micrographs of actin filaments decorated with smooth muscle S1 (Whittaker *et al.* 1995) and electron paramagnetic resonance studies of S1 introduced into muscle in rigor (Gollub *et al.* 1996). In both cases, addition of exogenous MgADP induced a *ca*. 23° rotation in the lever arm of myosin. Such a rotation would result in a reversal of the powerstroke from the rigor position and so suggests that ADP release in smooth, but not in skeletal, muscle provides an extra component to the power stroke of the cross-bridge cycle (Whittaker *et al.* 1995).

Following these structural observations, we found that addition of MgADP to permeabilized smooth muscle in the nucleotide-free rigor state led to a reduction in rigor force and stiffness (figure 6). These findings were in contrast to a previous study in which MgADP produced an opposite, albeit small (*ca*. 1%) increase in rigor force (Dantzig *et al.* 1999). The difference probably reflects the distinct experimental protocols used. We photolytically liberated ADP from caged ADP whereas the other study allowed the MgADP to diffuse into the fibre, which may have resulted in inhomogeneity in the ATP to ADP concentration ratio. The 6% decrease in rigor force in phasic smooth muscle observed (Khromov *et al.* 2001) was close to the theoretical estimate that considered compliance, the distance between dense bodies, and the estimated amplitude of MgADP-induced decrease in rigor force (10–12%) that would cause a *ca*. 23° rotation of the lever arm (Dantzig *et al.* 1999). The decrease in stiffness that accompanied the MgADP-induced decrease in rigor force was no greater than the changes expected as a result of the non-cross-bridge related part of the total compliance (and hence was unlikely a

result of cross-bridge detachment). Based on the relatively high MgADP concentration in normal smooth muscle and the high affinity of cross-bridges for MgADP makes it unlikely, based on thermodynamic grounds, as ADP release would have to occur against a gradient and would absorb, rather than release energy (Gollub *et al.* 1996; Khromov *et al.* 2001). Therefore, there is insufficient evidence as to whether the effects of exogenously added MgADP on nucleotide-free cross-bridges can necessarily suggest that the ADP release step makes it a significant contribution to force maintenance in smooth muscle (Khromov *et al.* 2001).

6. PHYSIOLOGICAL RELEVANCE OF ADENOSINE DIPHOSPHATE BINDING IN SMOOTH MUSCLE

The characteristic high economy of force maintenance in smooth muscle can be largely attributed to the high affinity of MgADP to unphosphorylated cross-bridges in the positively strained state as well as by a possible cooperative mechanism, whereby unphosphorylated cross-bridges attach to actin (Somlyo *et al.* 1988). We need to understand how cross-bridges with MgADP dissociation constants in the 0.5–5.0 μM range are modulated by cytosolic MgADP concentrations in the 50–150 μM range. It is also worth noting that the concentrations of CP (2–4 mM; Butler & Davies 1980) and CK (63 IU g^{-1} wet weight; Khromov *et al.* 1995) are low in tonic smooth muscle, being 10- and 100-fold, respectively, less than in striated muscle. This plus the paucity of mitochondria in smooth muscle will favour a low phosphorylation potential.

Modulation of cross-bridge behaviour *in situ* by an ADP concentration range that is so much greater than its dissociation constant arises by virtue of competition between ATP and ADP (Warshaw *et al.* 1991; Khromov *et al.* 1996). Thus we know the affinity of cross-bridges in rigor for ADP differs from the apparent affinity in the physiological state where 1–2 mM ATP is present in the cytosol (reviewed in Khromov *et al.* 1998*b*). If we consider simple competitive inhibition between 2.0 mM MgATP (K_m *ca.* 100 μM (Iino 1981; Arner & Hellstrand 1985)) and 100 μM MgADP ($K_i = 1\mu M$), ADP will decrease the rate of an ATP-mediated reaction sixfold (Khromov *et al.* 1995).

Kinetic and equilibrium constants of nucleotide binding to cross-bridges measured *in situ* generally agree well with those determined *in vitro*. For example, solution studies (Gollub *et al.* 1996; Cremo & Geeves 1998) show high affinity ($K_d = 5$ μM) of MgADP for the non-phosphorylated complex of chicken gizzard S1 and actin. These studies also report that the association constant of ATP for actomyosin is close to values obtained in skinned smooth muscle preparations (Nishiye *et al.* 1993; Khromov *et al.* 1996). Such comparative data and those reported elsewhere in this review give confidence in interpreting our *in situ* data in terms of their physiological significance.

We have also compared nucleotide interactions with cross-bridges in phasic and tonic smooth muscle. The apparent second-order rate constant of ATP-induced cross-bridge detachment from rigor in the absence of Ca^{2+} was significantly higher in the phasic ($9.5 \times 10^4\ M^{-1}\ s^{-1}$) than in the tonic ($3.0 \times 10^4\ M^{-1}\ s^{-1}$) muscle (Khromov *et al.* 1996). This, combined with a fivefold lower affinity of phasic smooth muscle actomyosin for ADP, results in a 15 times less pronounced dependence of the relaxation kinetics on MgADP in phasic type smooth muscles.

A characteristic of smooth muscle physiology is high force maintenance by an AM·ADP state that has relatively low RLC phosphorylation. Slow ADP dissociation from AM·ADP is also responsible for low shortening velocity. These properties are most prominent in tonic smooth muscles such as in blood vessels which maintain a high resting tone or maintain prolonged changes in blood flow. As noted above, distinct properties of tonic and phasic smooth muscle myosins relate to their myosin isoforms (Horiuti *et al.* 1989). The major differences in nucleotide binding and release kinetics are thought to reflect the presence (in phasic) or absence (in tonic) smooth muscle myosin of a seven amino acid insert in the myosin heavy chain (Lauzon *et al.* 1998). This is also suggested by correlations between muscle mechanical properties and isoform distribution (reviewed in Somlyo 1993). Distinct cross-bridge properties could also reflect differences in LC_{17} isoforms (Malmqvist & Arner 1991; Matthew *et al.* 1998) or in the variable expression of these isoforms (reviewed in Somlyo 1993).

7. PERSPECTIVE

It was in the early 1970s that smooth muscle myosin was shown to be filamentous (Rice *et al.* 1971; Somlyo *et al.* 1971; Ashton *et al.* 1975) and that actin and myosin filaments plus Z-disc-like dense bodies formed mini-sarcomeres (Bond & Somlyo 1982), giving strong support for smooth muscle showing properties consistent with the sliding filament hypothesis and cycling cross-bridges (Somlyo *et al.* 1988) already established for skeletal muscle. The overall picture of smooth muscle myosin regulation is, however, much more complex than that of skeletal muscle (Somlyo & Somlyo 2003), demanded in all probability by the multitude of distinct cellular and tissue specific functions involving smooth muscle. Nevertheless, as we have reviewed here, ADP release from myosin cross-bridges is regulated by several molecular processes and is a central parameter in control of smooth muscle contraction and force maintenance. It is also evident, in part from the papers of this discussion meeting, that ADP bound to myosin in the presence of actin has a multifaceted role in the control of contraction and force maintenance within the wider myosin superfamily.

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GLOSSARY

ADP: adenosine diphosphate
AM·ADP: strongly bound actomyosin-ADP cross-bridge state
AM: actomyosin nucleotide-free cross-bridge state
ATP: adenosine triphosphate
Caged ADP (ATP): $P^{2(3)}$-1-(2-nitrophenyl)ethyl ester of ADP (ATP)
3′-deac-edaADP: 3′-*O*-{-[2-(7-diethylaminocoumarin-3-carboxamido)ethyl]carbamoyl}ADP
CK: creatine kinase
CP: creatine phosphate
HMM: heavy meromyosin
IU: international units
LC_{17}: 17 kD light chain
mant-ATP: 2′(3′)-*O*-(*N*-methylanthraniloyl)ATP
MDCC-PBP: A197C phosphate binding protein adduct with *N*-(2-[1-maleimidyl]ethyl)-7-diethylamino-coumarin-3-carboxamide
MgADP: magnesium adenosine diphosphate
P_i: phosphate
P_{max}: maximum isometric force
Rbl: rabbit bladder
Rfa: rabbit femoral artery
RLC: regulatory light chain
S1: subfragment 1
V_0: velocity of unloaded shortening